Experimental methods for 3D structure determination

Josef Houser

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S1004 Methods for structural characterization of biomolecules

Quick reminder – structure hierarchy

	Protein	DNA
Primary	Sequence (aminoacids, N-term - C-term)	Sequence (nucleotides, 5`- 3`end)
Secondary	α-helix, β-sheet, turns, loops (rotation along torsion angels Ψ and Φ)	Watson-Crick base pairing (A-T, C-G)
Tertiary	3D organization of secondary motives	A-form, B-form, Z-form
Quarternary	oligomerization	nucleosomes

3D structure = tertiary.

Typically we also gain also primary, secondary and quarternary (not always) structure in one experiment

Methods

Nuclear magnetic resonance
= NMR

Cryo-electron microscopy
= Cryo-EM

Crystallography
(diffraction methods)

X-ray
neutron
electron

Methods

Nuclear magnetic resonance
= NMR

Cryo-electron microscopy
= Cryo-EM

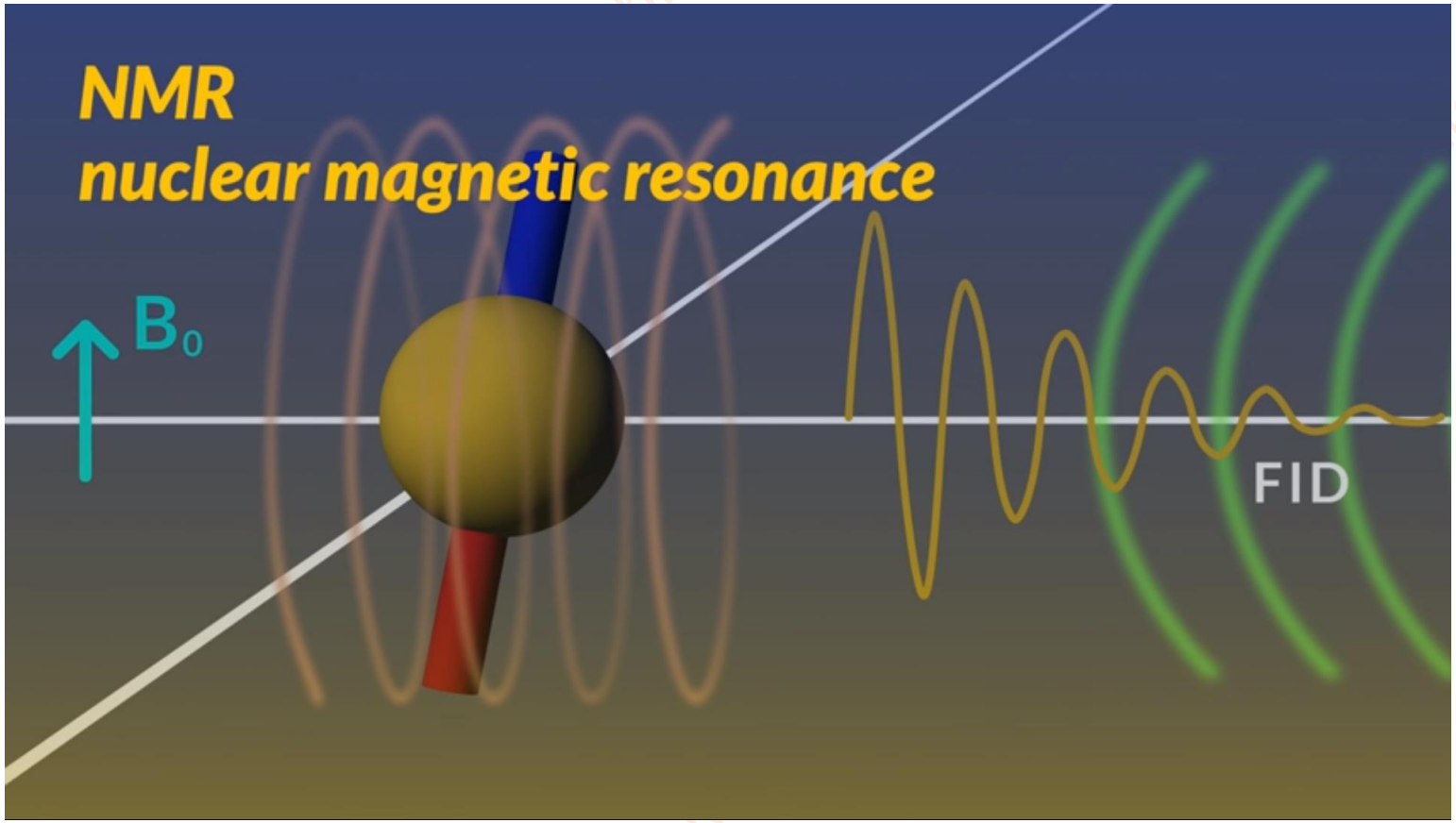
Crystallography
(diffraction methods)

- High resolution up to individual atoms
  1Å ≈ 0.1 nm ≈ length of covalent bond
- Results are x, y, z, coordinates of atoms position
- Require expensive instruments
- Nontrivial principles and data analyses

Methods

	NMR	Cryo-EM	Crystallography
sample	in solution	in solution	**crystal**
sample concentration	high (mM)	low (pM)	average (µM)
interact with	nuclei	electrons	depends on radiation type
size of molecule	**small (< 40 kDa)**	**big (> 100 kDa)**	both
protein complexes	no	**yes**	with limits
dynamics	**yes**	no	no
resolution	high (~ 1Å)	reasonable (2.5Å)	high (1Å)
duration of experiment	days	hours	minutes (days for neutron crystallography)
high throughput	no	no	**yes** (X-ray crystallography)

NMR



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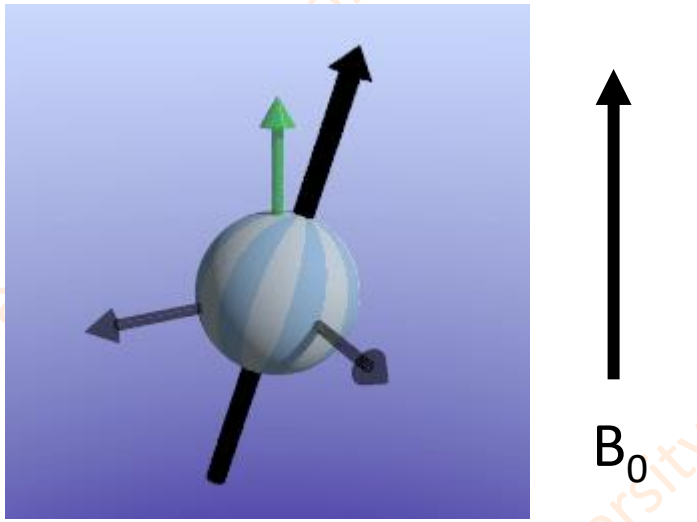
NMR

Sample is placed inside a strong magnetic field.

Nuclei in the sample are oriented along the magnetic field and start to spin = precession.



Important!

^{1}H, ^{13}C, ^{15}N, ^{19}F isotopes needed in the sample

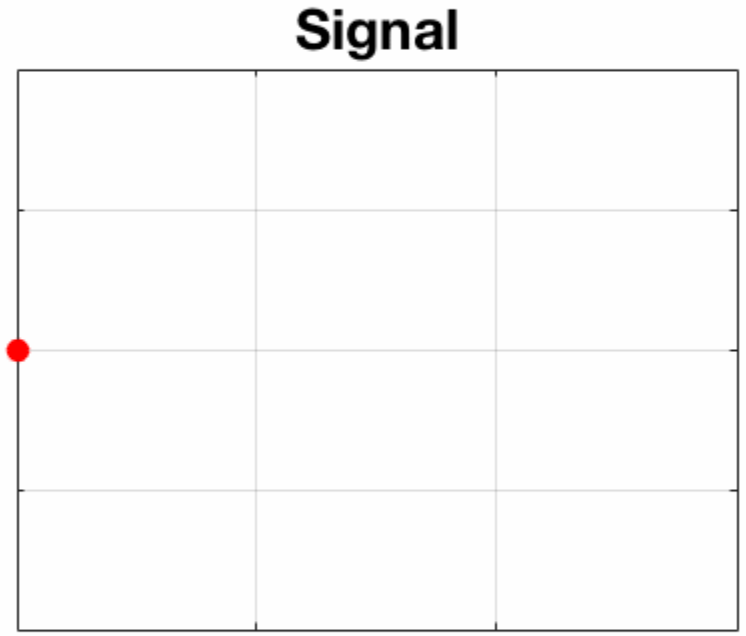
NMR

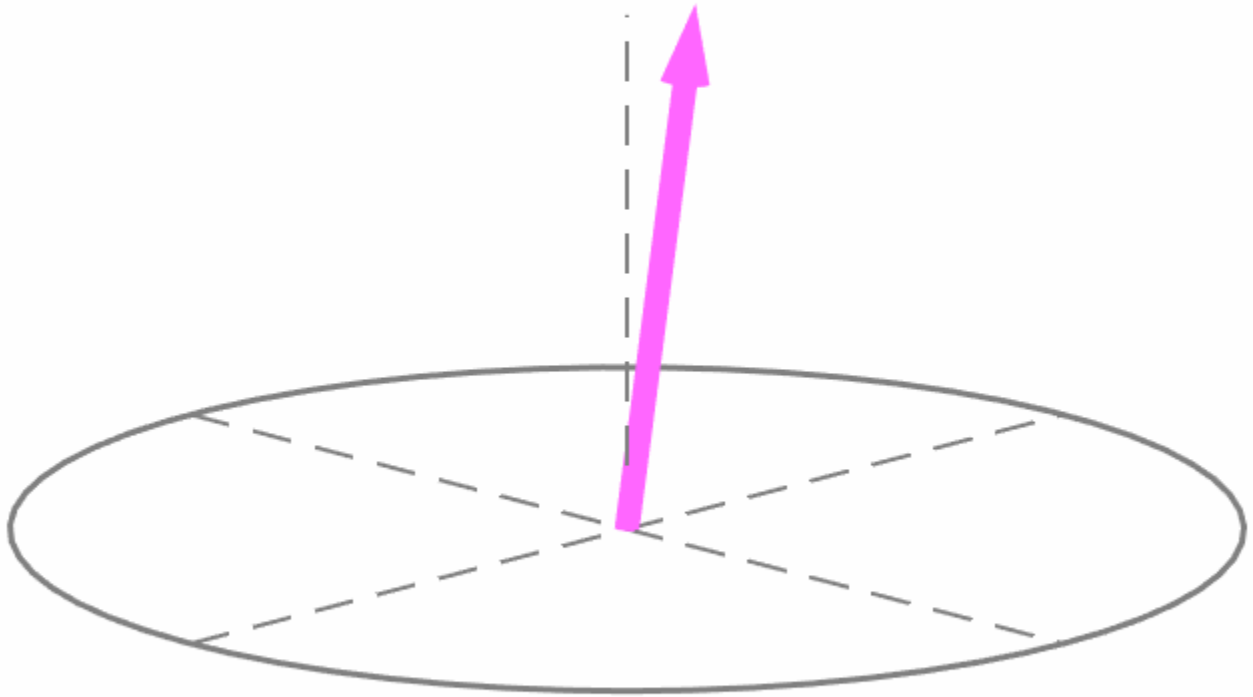
When activated by a radio frequency wave, the precession axis deviates and than aligns back.

Allows to detect the frequency of precession and the time of return

(= relaxation time)

Depending on the nucleus surrounding the frequency of precession and relaxation time vary.


Signal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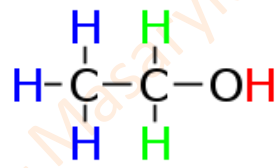
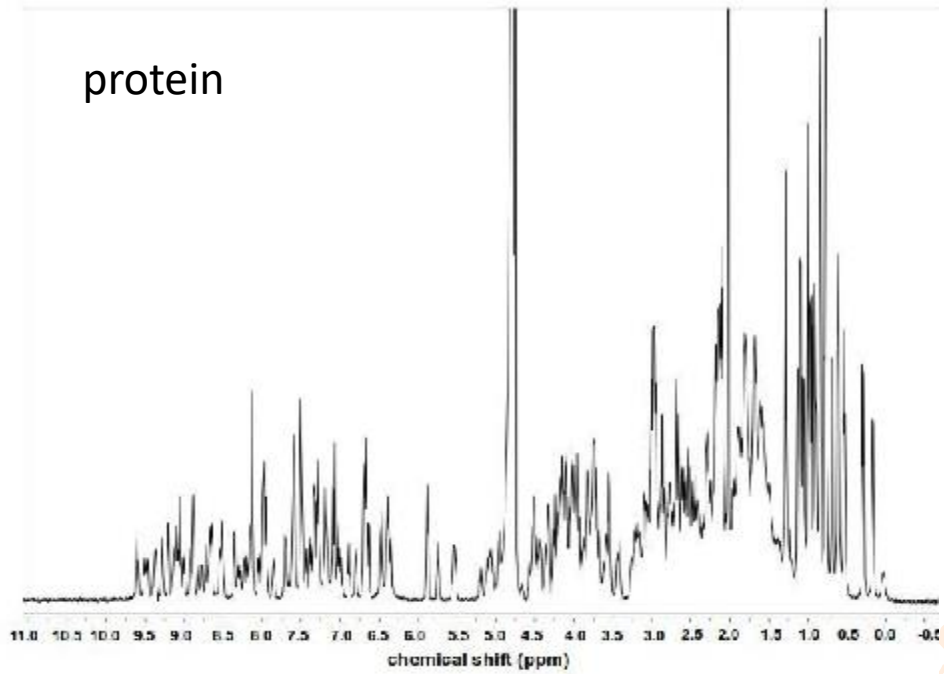
NMR

Ethanol



protein



1D experiment is sufficient for small organic molecules

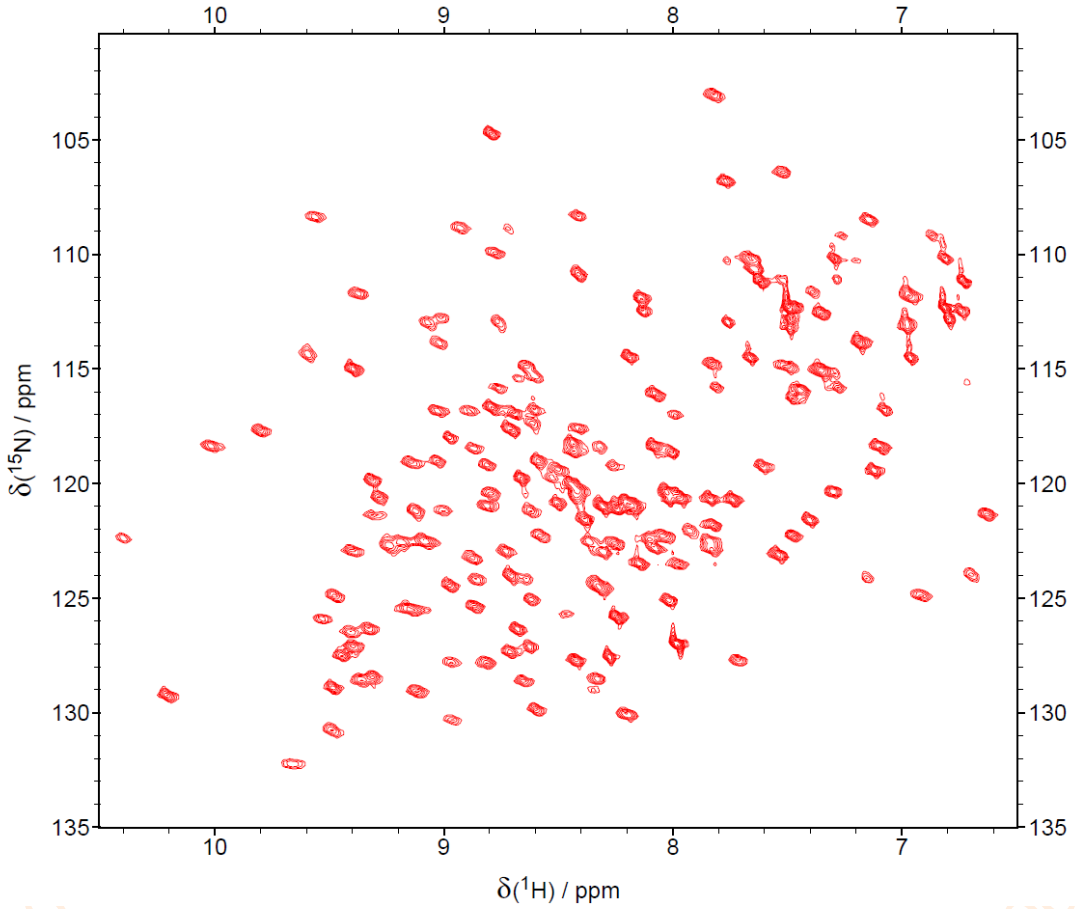
It is too crowded for biomacromolecules

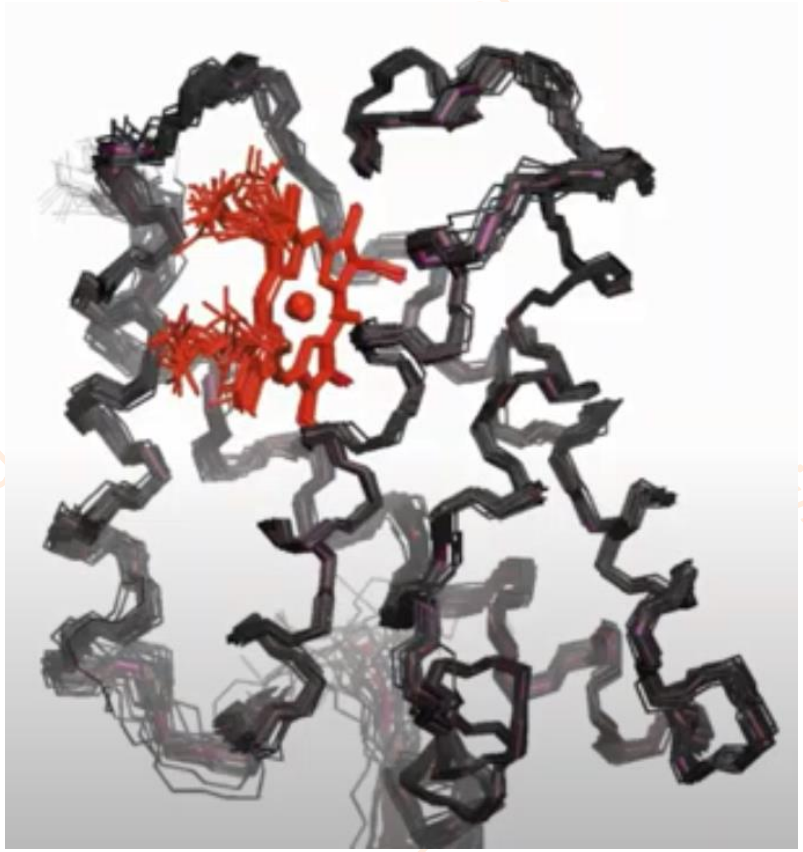
NMR

For proteins (DNA) – more complex experiments and their combination needed

- Series of activation pulses – signal generated by the same nucleus will corelate
- Specific series of pulses enable the transfer of magnetization
  - To atoms bound with covalent bonds (J-coupling)
    - Sequence, the side chains orientation
  - To atoms in close vicinity without covalent bond (NOE = nuclear Overhauser effect)
    - Tertiary structure

NMR





Human hemoglobin PDB:2M6Z

NMR

Basic principle	Measures precession of nuclei in strong magnetic field
Sample requirements	In solution at high concentration (mM)
Pros	Can detect dynamics of the molecule – sees also moving parts Sample in solution „natural environment“
Cons	Only some isotopes are compatible – special sample preparation For smaller proteins (< 40 kDa)

https://youtu.be/RZLew6Ff-JE

https://youtu.be/Sn3dNMv-67k

https://youtu.be/Enda859ftFQ

http://dx.doi.org/10.1016/j.ab.2016.05.006

Cryo-electron microscopy

Recently very popular and developing method

Nobel price in 2017

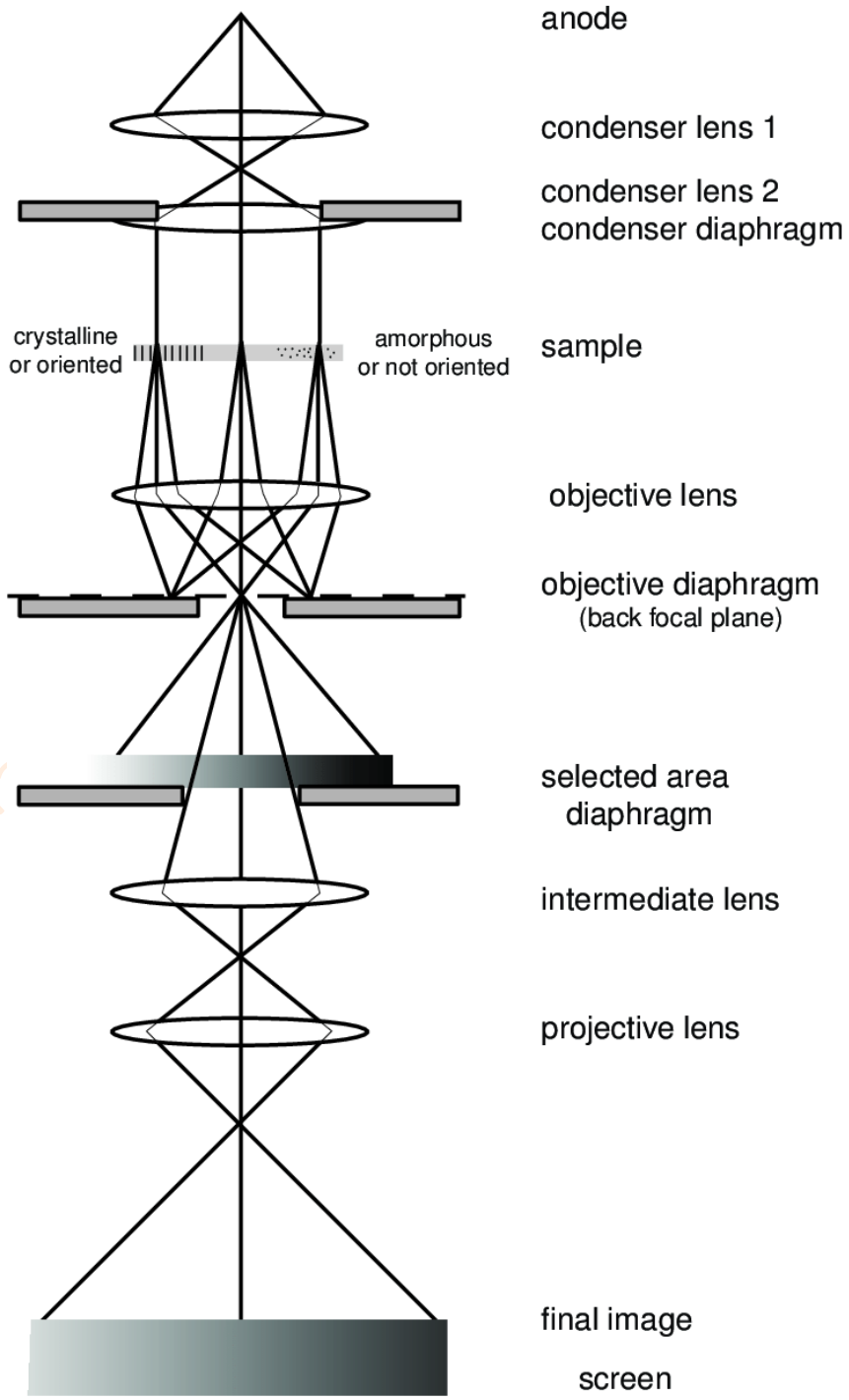
Produced in Brno – Thermofisher (Slatina)
Tescan (Kohoutovice)

Suitable for bigger molecules (> 100 kDa)
and complexes

Cryo-electron microscopy

- Electron beam interacts with sample molecules
- In vacuum
- Coils with magnetic field serve as lenses
- The image of the sample is bigger

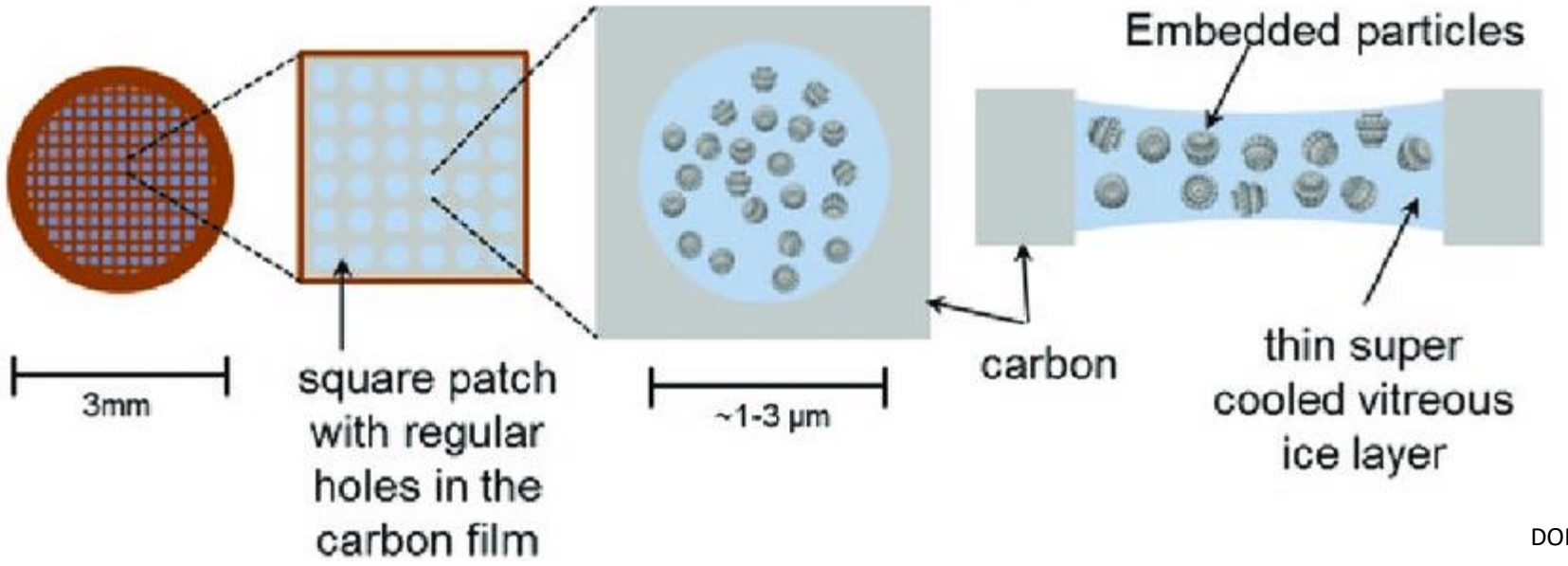
  (magnification 1: 5 000 000) and inverted



Cryo-electron microscopy

Sample preparation

- Extraordinary sample purity is needed
- Loading on the grid
- Vitrification (flesh freeze in liquid etane -88 °C)
- Needs to be thin - < 500 nm

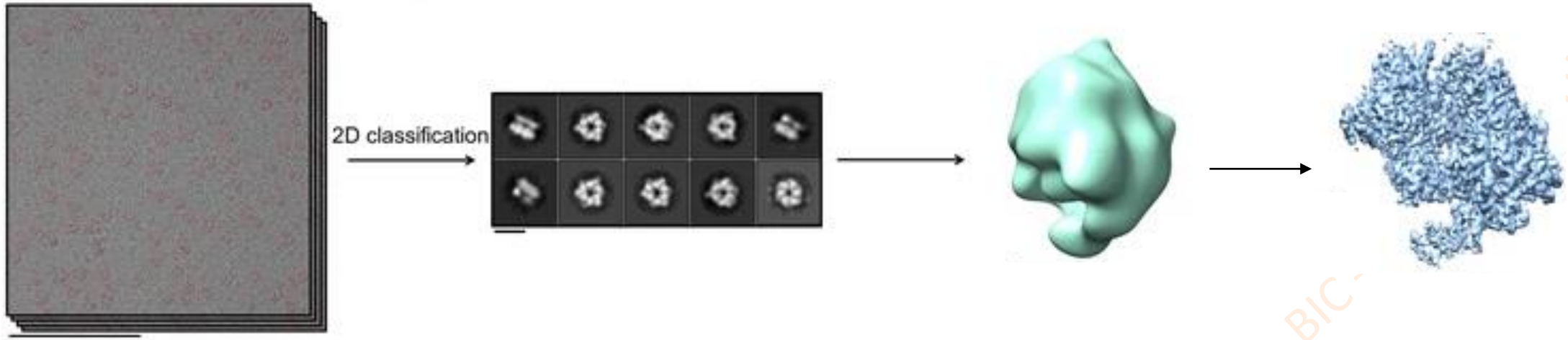


DOI: 10.1007/978-1-4939-7033-9_28

Cryo-electron microscopy

Data analysis:

- Identification of the molecule of interest
- Arranging into groups and averaging – 2D classification
- Assessment of orientation
- Combining to 3D

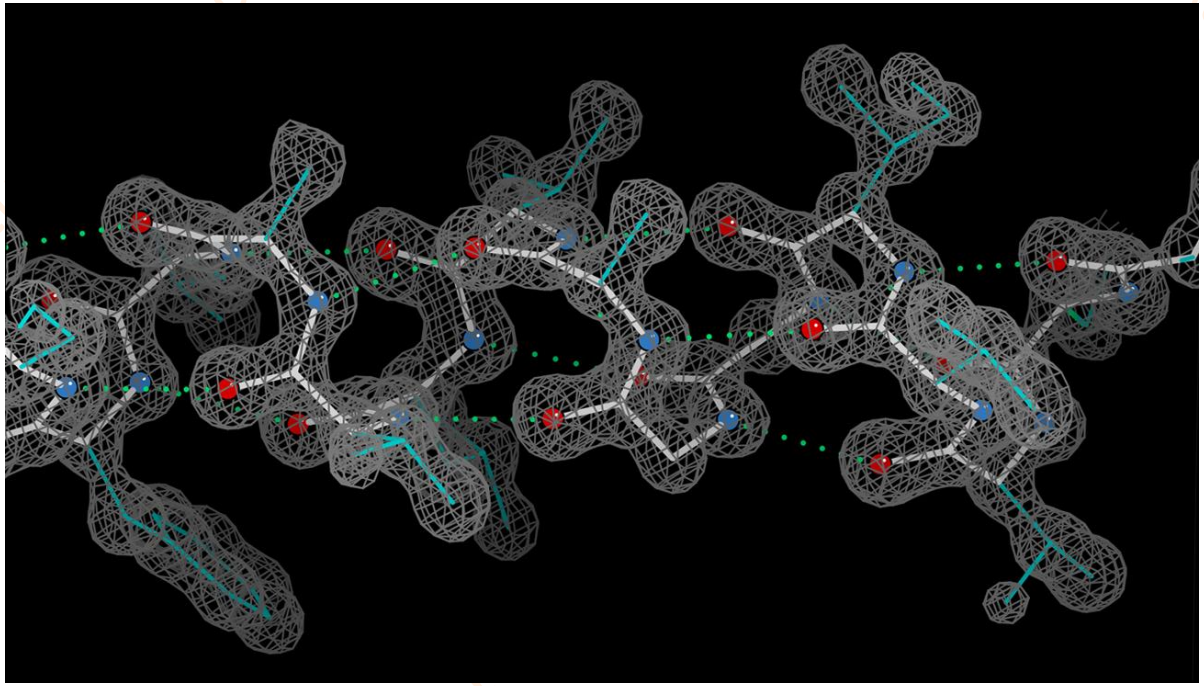


DOI: 10.7554/eLife.34257

Cryo-electron microscopy

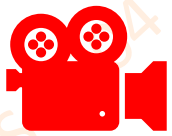
Result:

- Electron density map in high resolution (around 3Å, improving)



Cryo-electron microscopy

Basic principle	Interaction of electrons with atoms of the sample.
Sample requirements	Low concentration, high purity, vitrification
Pros	Excelent method for protein complexes, viral particles
Cons	Smaller proteins (< 100 kDa) do not produce enough signal Demanding data analyses (months)



Grant Jensen CALTECH youtube course

https://youtu.be/ljTEG-B-kGc

https://youtu.be/t4hhdgJADE8

https://doi.org /10.1007/978-1-4939-7033-9_28

https://doi.org/10.1016/j.abb.2014.11.011

Diffraction methods

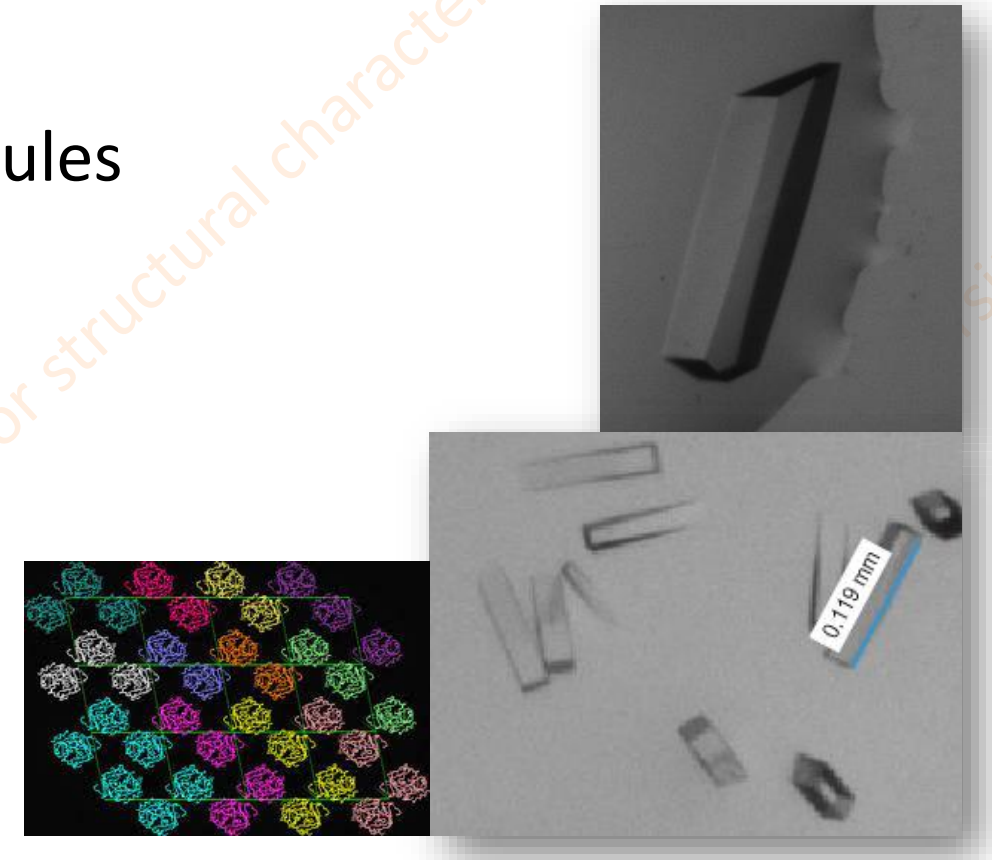
- Diffraction happen, when the wavelenght of the radiation is at the similar range as the object (resolution)

- Not only diffraction:
  - Nothing – radiation goes through the sample
  - Absorbtion → radiation damage
  - Inelastic scattering = change of the particle energy → noise
  - Elastic scattering = the particle energy is conserved → diffraction signal

Diffraction methods

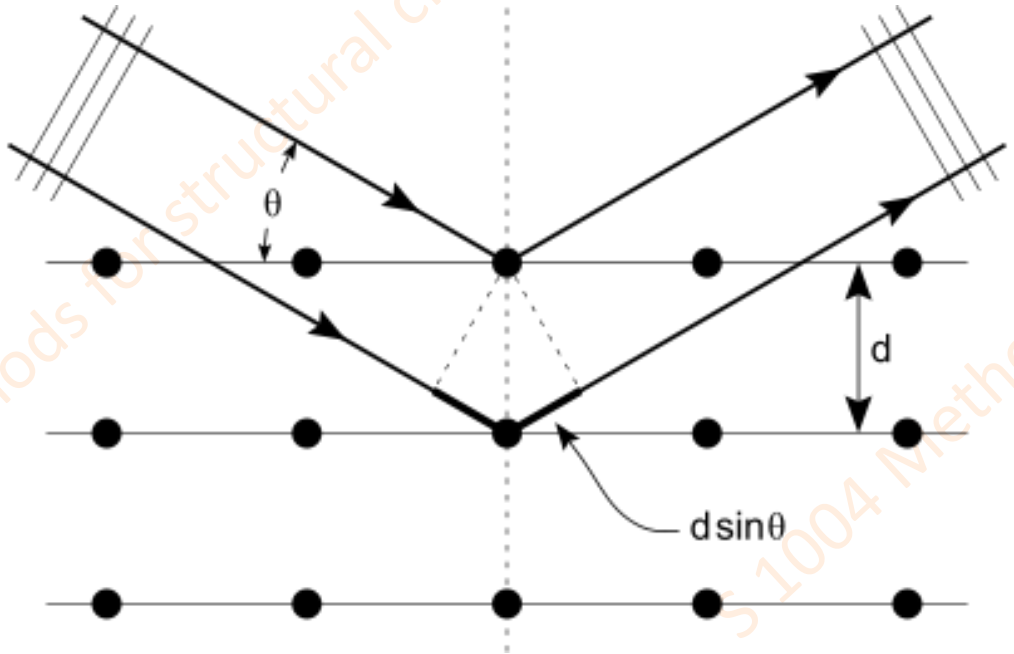
- Elastic scattering from single particle also happens, but the signal is very weak and noisy

- To increase the signal – CRYSTAL
  - Periodic arrangement of protein molecules with identical orientation
  - Difficult to obtain
  - Fragile



Diffraction methods

- Signal is enhanced on crystal by a constructive addition of elastically diffracted waves
- Waves need to stay in the same phase
- The extra path equals to integer multiple of λ



$$n\lambda = 2d\sin\theta$$

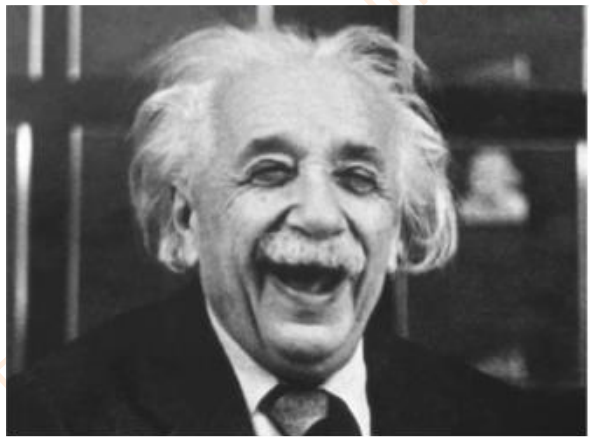
Bragg`s law

Diffraction methods

- If we want atomic resolution, we need to choose the radiation with a wavelength at the same range

The radiation can be: photons (X-ray)
neutrons
electrons

} particles obey quantum physics



Diffraction methods

	Photons (X-ray)	**Neutrons**	**Electrons**
Scattered by	electrons	nuclei	both
Scattering factor of elements	dependent on Z	independent on Z	dependent on Z and atomic charge
Speed	c = 299 792 458 $m.s^{-1}$	≈ 2600 $m.s^{-1}$	≈ 6 000 000 $m.s^{-1}$
Rest mass	none	1.675 x 10^{-27}kg	9.1091 x 10^{-31} kg
Energy	7 – 17 keV	0.1 meV – 0.5 eV	100 – 300 keV
Wavelength	0.07 – 0.17 nm	0.01 – 3 nm	2 – 4 pm
Crystal size	Medium (µm)	Big (mm)	Small (nm)

X-ray diffraction

- The oldest and best established diffraction method
- Uses synchrotron radiation
- Quick data collection (minutes)
- High throughput

X-ray diffraction

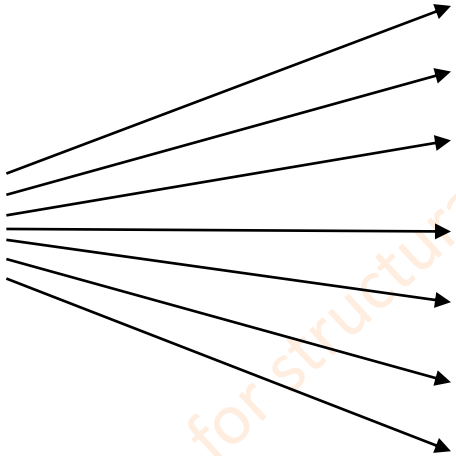
X-ray = photons

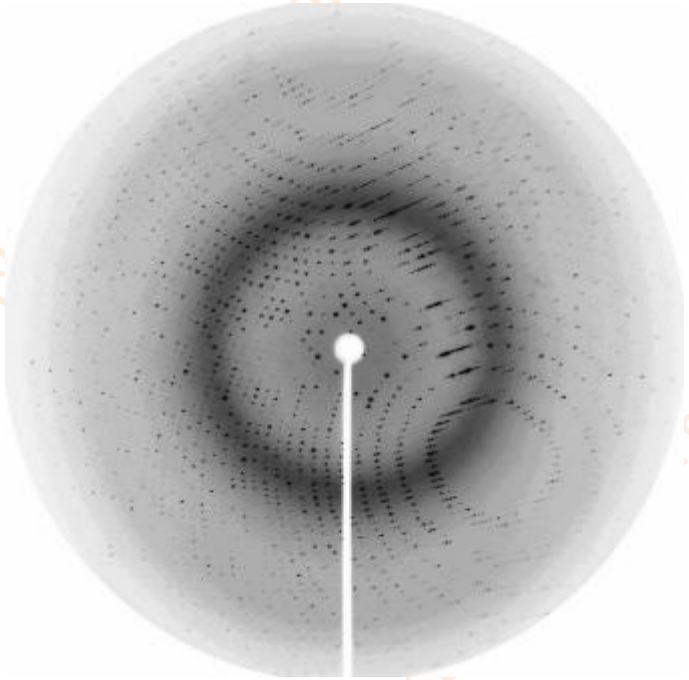
Elastic scattering

Diffraction pattern

Synchrotron

Crystal



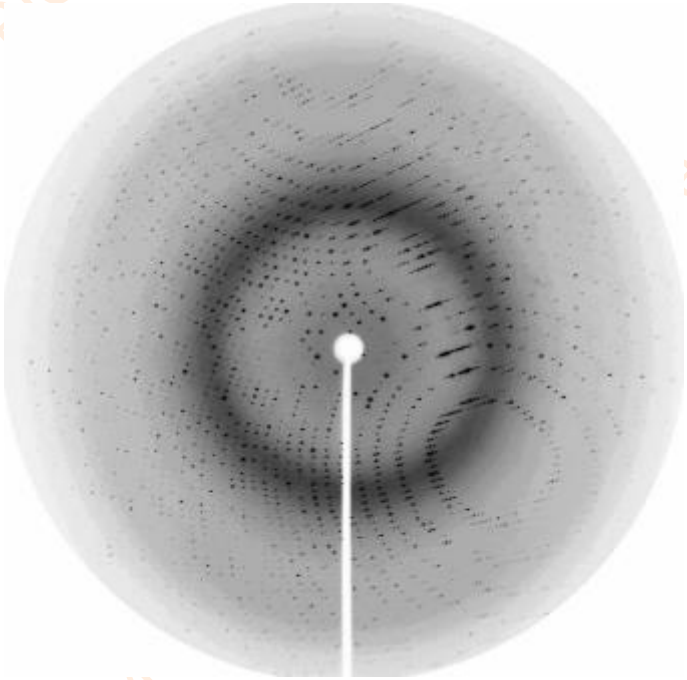


To prevent radiation damage – measurement at cryogenic temperatures (–196 °C)

Oscilation during data collection typically 0.1°

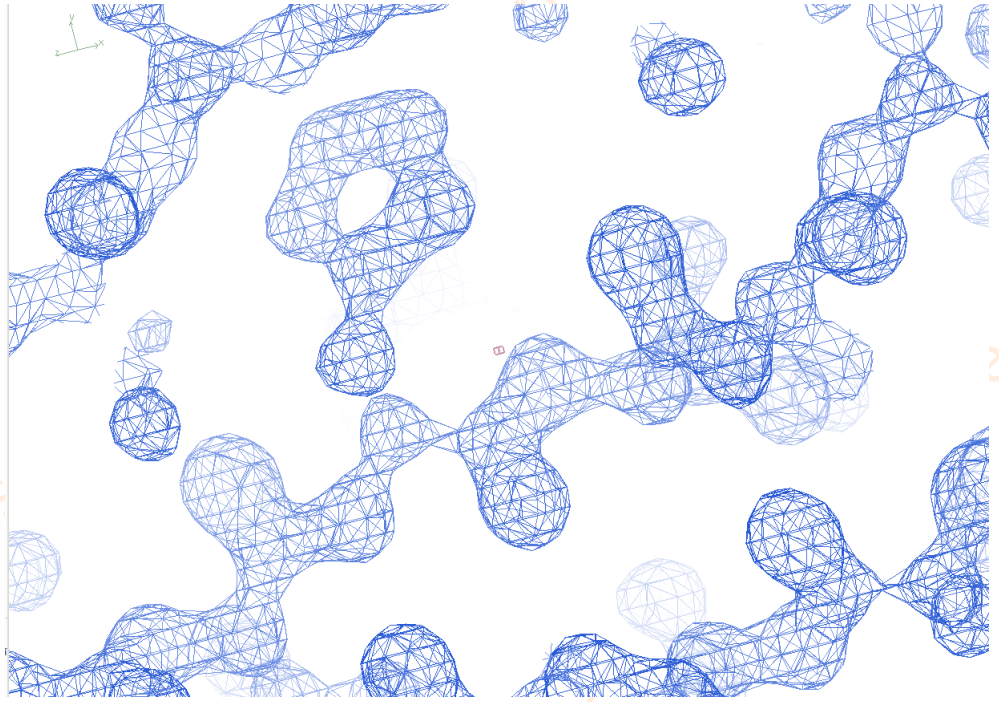
X-ray diffraction

Diffraction pattern



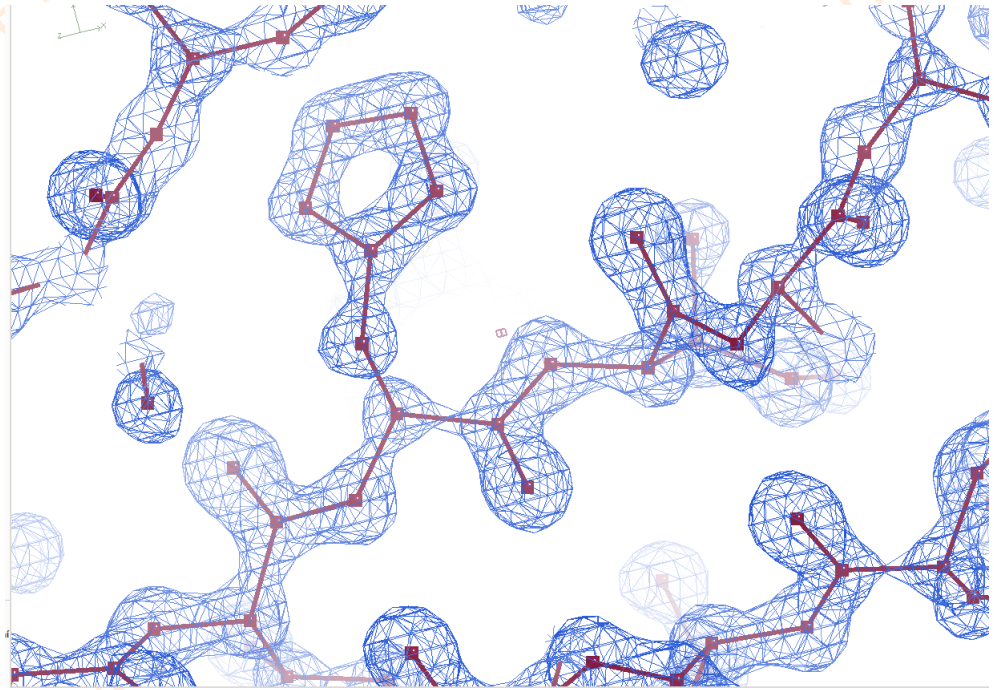
Fourier transform

Electron density map



X-ray diffraction

Protein model inside the electron density map



Refinement

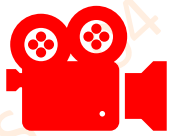
- Several rounds
- Improves the model

Validation

Deposition to the database

X-ray diffraction

Basic principle	Elastic scattering of x-ray photons from electron cloud of the sample arranged in crystal
Sample requirements	Crystal (medium size, µm) Typically cryogenic temperatures
Pros	Quick data collection and analyses, high throughput, automation
Cons	Crystals are sometimes difficult to obtain H atoms are not visible even at high resolution Costly instrumentation (synchrotron)



https://youtu.be/QuCRBxjk3fg

https://doi.org/10.3390/molecules25051030
https://doi.org/10.1007/978-1-60327-159-2_3

Neutron diffraction

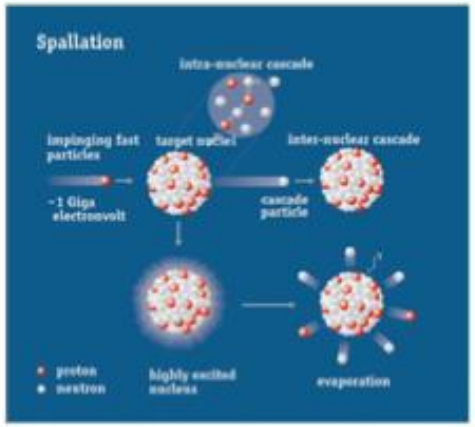
- The source of neutrons is nuclear reaction
- Slow data collection (days)
- Requires huge crystals
- Can visualise hydrogens

Neutron diffraction

How to produce free neutrons?

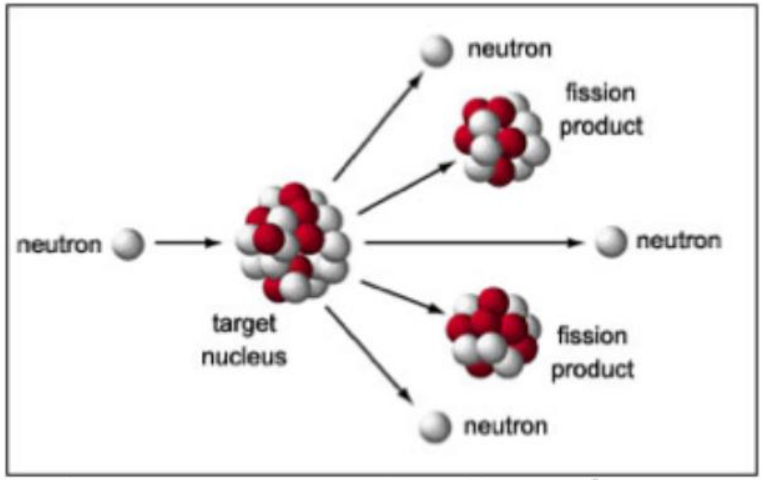
1. Radioactive decay
2. **Fission**
3. **Spallation**
4. Fusion

Particles are typically protons, targets include Ta, W, U, Hg



Spallation production: ~ 60 n.proton^{-1}

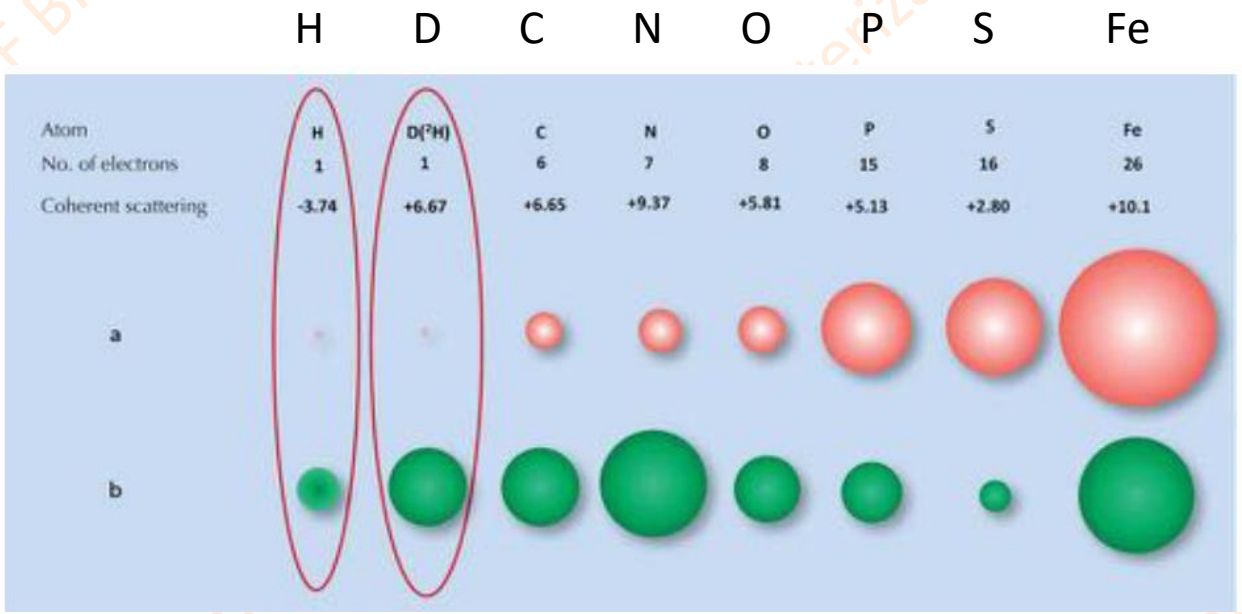
$^{235}U + n_{slow} \rightarrow 2$ fission fragments + 2.5 n_{fast} + 180 MeV



Reactor production: ~ 1 n.decay^{-1}

Neutron diffraction

Scattering factor independent on Z (proton number of element)

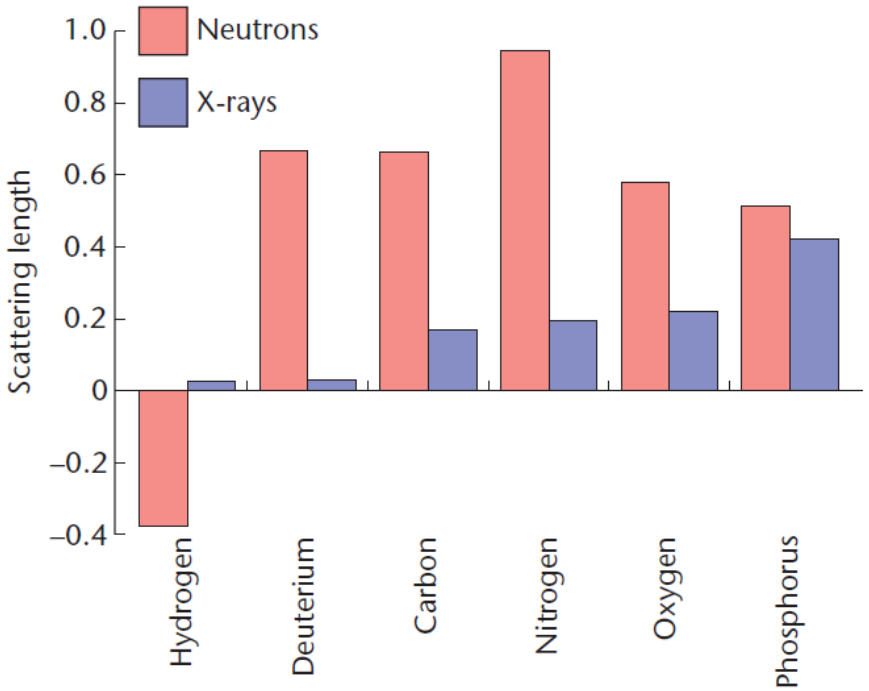


X ⇨ **X-ray do not see H**

N ⇨ **Neutrons can see H**

Neutron diffraction

Scattering factor independent on Z (proton number of element)



One catch – H has negative scattering lenght

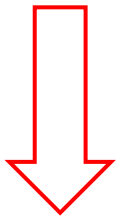
CH_2 group cancel each other out

Sample deuteration needed

Deuteration also to improve signal noise ratio (H has large incoherent scattering)

Neutron diffraction

Low energy

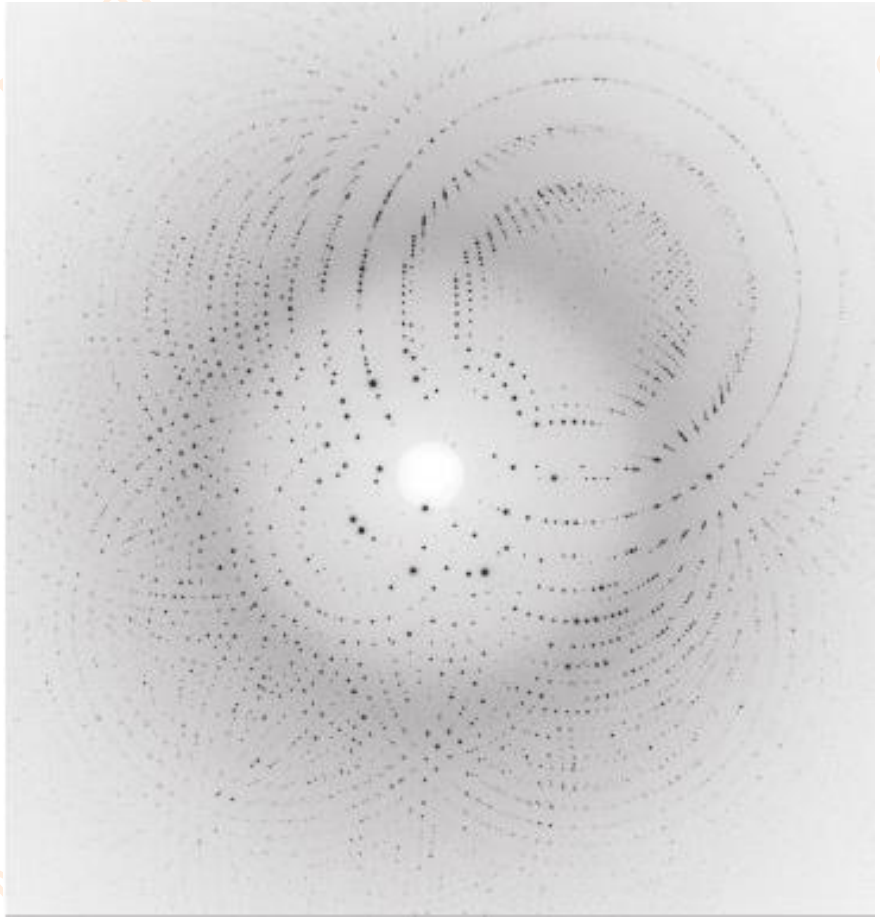


No radiation damage

Measurement at room temperature for very long time

(neutron flux is low, so long expositions is needed to have reasonable signal intensity)

Neutron diffraction



Laue diffraction

- Compensation for a long exposition time
- Use neutrons of multiple wavelenghts at the same time
- Intersection of the detector with more Ewalds spheres on the same picture
- Allows to used higher angles of oscilation
- Requires special data processing

Neutron diffraction

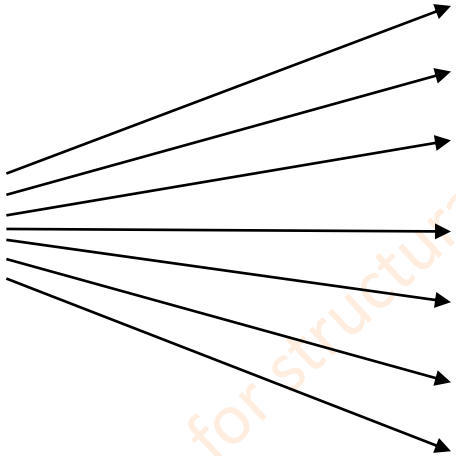
Neutrons

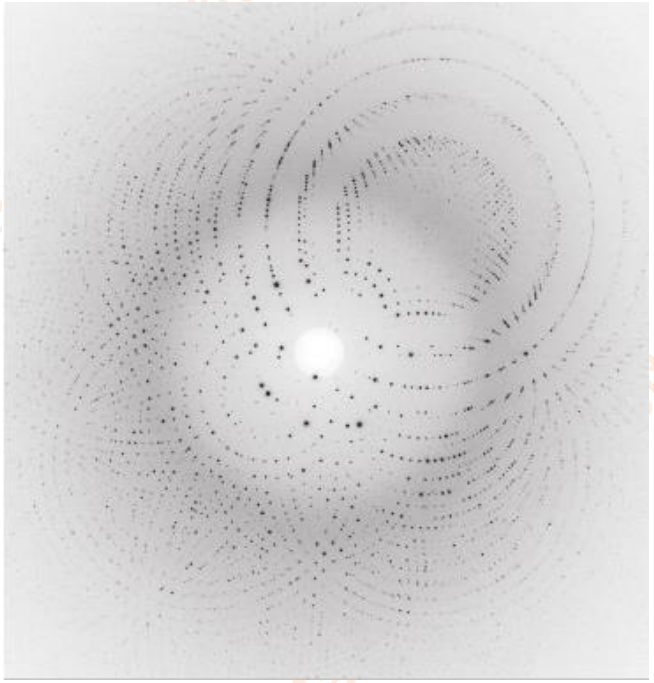
Elastic scattering

Diffraction pattern

Nuclear reactor

Crystal



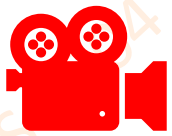


Radiation damage is low – measurent at room temperature

Oscilation during data collection up to 7°

Neutron diffraction

Basic principle	Elastic scattering of neutrons from nuclei of the sample arranged in crystal
Sample requirements	Crystal (big, mm) Typically at room temperature
Pros	Visible hydrogens – exact study of hydrogen bonds, protonation states
Cons	Huge crystals are needed – even more difficult to obtain Requires deuteration of the sample – expensive Very limited access to radiation sources

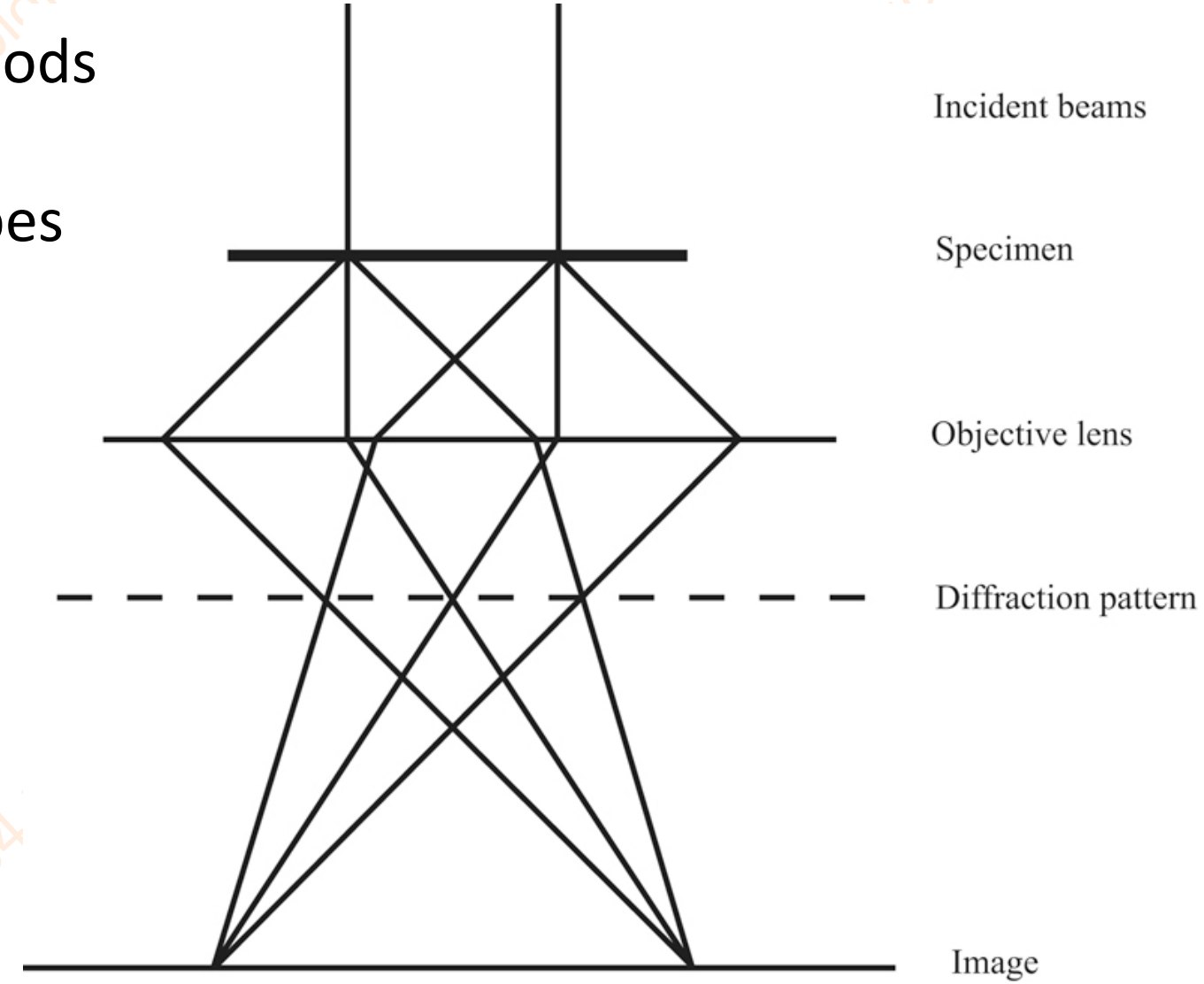


https://youtu.be/Ep8qWJhS894

https://doi.org/10.1002/9780470015902.a0003045.pub2

Electron diffraction

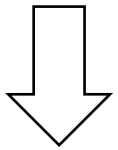
- The youngest of diffraction methods
- Performed at electron microscopes
- Needs only tiny crystals (nm)



Electron diffraction

- Why to use electrons?

- They strongly interacts with matter
  – elastic scattering represents 25 % of scattered electrons

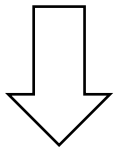


- Needs only sub-micrometer crystals – perfect for systems that do not form bigger crystals (membrane proteins)

Electron diffraction

- Why not to use electrons?

- They strongly interacts with matter
  – problem of multiple scattering of 1 electron inside the sample



- Difficult to take into account in data processing – introduces errors
- Increases with sample thickness – ideal size 100-200 nm

Electron diffraction

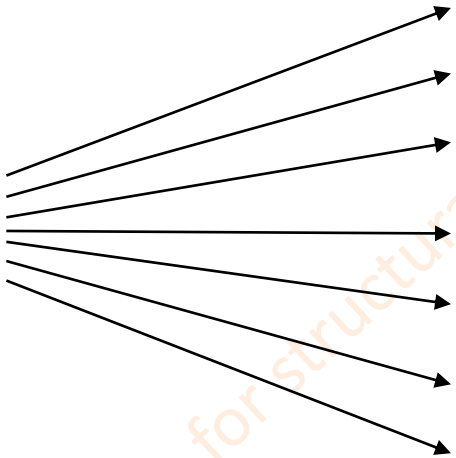
Electrons

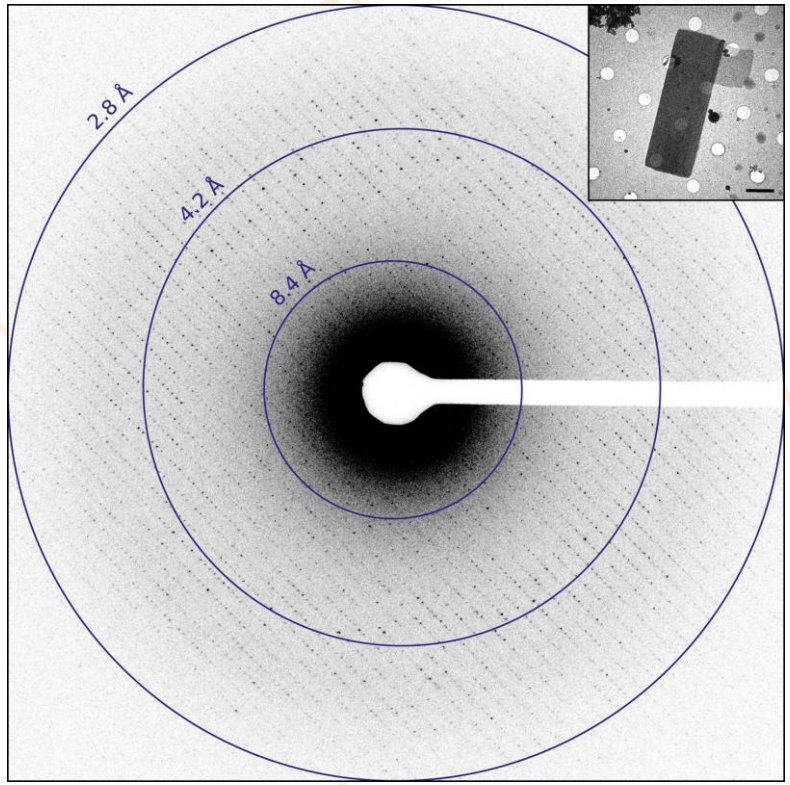
Elastic scattering

Diffraction pattern

Electron microscope

Crystal





DOI: 10.7554/eLife.03600

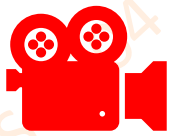
Oscilation during data collection usually 0.1°

Sample holder allows to rotate only 80° of the crystal

Electron diffraction

Basic principle	Elastic scattering of electrons from the sample arranged in crystal
Sample requirements	Crystal (tiny, nm) Performed in vacuum
Pros	Better accesibility of cryo-electron microscopes Microcrystals are easier to produce
Cons	Secondary scattering of electrons

Still in development



https://youtu.be/s5lWzf1FZB0

https://doi.org/10.1107/s2059798320016368

Biomolecular Interactions and Crystallography Core Facility

bic@ceitec.cz

bic.ceitec.cz

MUNI | CEITEC

CF Head

Josef Houser

- +420 549 492 527
- josef.houser@ceitec.cz